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LISTING OF DISCLOSURE AMENDMENTS

Please amend page 1 of the Disclosure as follows:

COMPOUNDS WITH ANTI-KS AND ANTI-HIV ACTIVITY

This application is a division of United States Patent Application Serial Number 09/494,500 filed January 31, 2000, which is a continuation of PCT/CA98/00731 filed July 30, 1998 designating the United States and claiming priority of United States provisional Patent Application Serial Number 60/054,543 filed August 1, 1997.

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to compounds which exhibit anti-KS and anti-HIV activity, pharmaceutical compositions and method of treatment thereof.

(b) Description of Prior Art

Kaposi's sarcoma (KS) is the most common tumour in AIDS subjects which afflicts high mortality (Friedman-Kien AE et al., 1990, *J Am Acad Dermatol* **22**:1237-1250). Less aggressive forms can also occur in non-AIDS subjects of the Mediterranean area and equatorial Africa as well as in renal transplant patients following treatment with immunosuppressive drugs (Friedman-Kien AE et al., 1990, *J Am Acad Dermatol* **22**:1237-1250). The pathogenesis and therapy of KS remain enigmatic (Bais C. et al., 1998, *Nature* **391**:86). For unknown reasons, occurrence of KS is higher in males than in females. For example, in the West, approximately 95% of AIDS-KS subjects are men. Although, hormonal dependence of KS has been demonstrated in the case of glucocorticoid and retinoid

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(Guo WX et al., 1996, *Am J Pathol* **148**: 1999-2008; Guo WX et al., 1995, *Am J Pathol* **146**: 727-734; Guo WX et al., 1995, *Cancer Res* **55**: 823-829), sex steroids do not seem to be directly involved in KS pathogenesis. Recently, Lunardi-Iskandar et al. (Lunardi-Iskandar Y et al., 1995, *Nature* **375**: 64-68) reported that the placental hormone human chorionic gonadotropin (HCG), displays anti-KS activity and prevents tumours in immunodeficient mice. This preliminary finding could

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Please amend page 3 of the Disclosure as follows:

Immunol Lett 44: 13-18) suggest the involvement of an "unconventional" mode of action for HCG in KS. In fact, they reported a biological activity for the β HCG, a notion which contradicts the generally accepted paradigm that the dimeric form of the hormone is required for triggering hormonal responses in classical target tissues. While stirring a controversy (Lunardi-Iskandar Y et al., 1995, Nature 377: 21-22; Berger P et al., 1995, Nature 377: 21; Rabkin CS et al., 1995, Nature 377: 21-22; Krown SE, 1996, New Engl J Med 335: 1309-1310), these findings raise intriguing and potentially novel issues.

There is reported in Nature Medicine (Vol. 4, No. 7, July 1998) that the anti-KS activity of crude hCG preparations is still a mystery.

It would be highly desirable to be provided with compounds which would exhibit anti-KS and anti-HIV activity.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide with compounds which would exhibit anti-KS and anti-HIV activity.

In accordance with one preferred embodiment of the present invention there is provided a compound having anti-KS and anti-HIV pharmaceutical activity which comprises an HCG-like inhibitory protein and fragments thereof, the protein and fragments thereof are isolated from a biologically active fraction of APL™-HCG ("APL™" is the commercial trade-name of the clinical-grade HCG sold by Wyeth-Ayerst), wherein said protein has a molecular weight of about 3,500 or of

about 13,000 Dalton, and wherein said protein and fragments thereof are adsorbed to polypropylene plastic supports, such as tubes or pipette tips among others.

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Please amend page 6 of the Disclosure as follows:

comprises administering to said patient a therapeutically effective amount of a protein of the present invention.

In accordance with another preferred embodiment of the present invention there is provided a method for the prevention, treatment and/or reduction of Kaposi's sarcoma and/or HIV expression in AIDS patients, which comprises administering to said patient a therapeutically effective amount of a pharmaceutical composition of the present invention.

In accordance with another preferred embodiment of the present invention there is provided a method to purify the compound or protein of the present invention, which comprises the steps of:

a) subjecting a biologically active fraction of APL™-HCG or urinary extract containing said compound or protein to a polypropylene plastic support for a time sufficient for adsorption of said compound or protein to occur; and
b) washing the support and releasing the adsorbed compound or protein therefrom.

In accordance with another preferred embodiment of the present invention there is provided a method of evaluating inhibitory activity of anti-KS and anti-HIV compound, which comprises by measuring AP1 gene activity.

In other embodiments, measuring of said AP1 gene activity is effected by measuring binding to DNA response element.

For the purpose of the present invention the following terms are defined below.

"HIP" : HCG-like Inhibitory Protein;

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"HPLC" : high-pressure liquid chromatography; and

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Please amend page 7 of the Disclosure as follows:

"APL™" : commercial trade name of the clinical-grade HCG sold by Wyeth-Ayerst, cat. # DIN 02168936.

The expression "derivatives and fragments thereof" is intended to mean any derivatives and fragments of the protein of the present invention which exhibit anti-KS and anti-HIV pharmaceutical activity effective for the prevention, treatment and/or reduction of Kaposi's sarcoma in AIDS patients. The derivatives may include one or more D-amino acids or non-natural amino acids. The derivatives and fragments are functional and substantially exhibit the biological activity of the protein of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the effect of HCG from different commercial sources on KS-Y1 cell proliferation;

Fig. 2 illustrates the fractionation and activity profile of APL™-HCG;

Fig. 3 illustrates the time-course effect of APL™-HCG on inhibition of AP-1 binding in KSY-1 cells;

Fig. 4 illustrates the purification of the HIP using reversed phase-HPLC;

Fig. 5 illustrates the bioassay of the collected fractions following HPLC separation;

Fig. 6 illustrates the analysis of fraction D by mass spectrometry; and

Fig. 7 illustrates the analysis of fraction A + B + C + E by mass spectrometry;

Fig. 8 illustrates the analysis of another low molecular weight fraction by mass spectrometry;

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Fig. 9 illustrates the effect of HIP on HIV expression; and

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Please amend page 9 of the Disclosure as follows:

cell growth, HCG radioreceptor binding and steroidogenic bioactivity. The Applicants' results demonstrate that the anti-KS activity resides among low molecular weight components, and not in *bona fide* (macromolecular) HCG. Interestingly, the Applicants have identified a transcription factor which may be the target for regulation by the anti-KS components. The Applicants have concluded that, as yet unidentified molecules, present in the commercial HCG preparations, are responsible for the growth inhibitory effects wrongfully attributed to HCG.

Surprisingly, and in accordance with the present invention, there is provided the identification of a purified HIP protein having anti-KS and anti-HIV pharmaceutical activity. This protein is an HCG-like inhibitory protein and is adsorbed to polypropylene plastic supports, and has an amino acid sequence selected from the group consisting of :

Ser-Lys-Glu-Pro-Leu-Arg-Pro-Arg-Glu-Arg-Pro-Ile-Asn*-Ala-Thr-Leu-Ala-Val-Glu-Lys SEQ ID NO:1;

and

Ala-Pro-Asp-Val-Gln-Asp-Lys-Phe-Thr-Arg-Gln-Ile-Met-Ala-Thr SEQ ID NO:2.

Sources of HCG

Two commercial HCG samples were tested. The first one, under the trade name of APL™, was provided by Wyeth-Ayerst, Montréal (Lot # C84662A was generously donated and cat. # DIN-02168936 was purchased), it should be emphasized that APL™ was used in the earlier

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studies (Lunardi-Iskandar Y et al., 1995, *Nature* **375**: 64-68; Gill PS et al., 1996, *New Engl J Med* **335**: 1261-1310). Two samples were also purchased from Sigma, St-Louis, Mo (lot # 26H 1040). Pure HCG dimer as well

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as α-HCG and β-HCG were obtained from NIDDK (Bethesda, MD). Recombinant HCG was obtained from Organon, Oss, the Netherlands. All HCG samples, previously stored lyophilised, were dissolved in PBS and frozen as aliquots.

Assessment of cell proliferation

The KS-Y1 (Lunardi-Iskandar Y et al., 1995, *Nature* **375**: 64-68) was isolated from an HIV-patient while the subline designated N-1506 (Lunardi-Iskandar Y et al., 1995, *Nature* **375**: 64-68) of the original KS-SLK cell line originated from an immunosuppressed subject (Herndier BG et al., 1994, *AIDS* **8**: 575-581). These cell lines were provided by Dr. Lunardi-Iskandar (N.I.H., Bethesda). The KS cells were passaged and the culture medium was changed every other day in presence or in absence of any of the HCG samples mentioned above for the indicated periods ranging from 24-96 hrs. ^{3}H-thymidine incorporation was measured as described (Guo WX et al., 1996, *Am J Pathol* **148**: 1999-2008; Guo WX et al., 1995, *Am J Pathol* **146**: 727-734) In most experiments, data are reported as means ± SEM of quadruplet determinations. Statistical analysis was determined by student t-test.

Fractionation of APL™ on SEPHADEX™ G-100

Three vials of APL™ (10 000 IU/vial) were pooled for fractionation by dissolving in 1.5 ml of 0.05 M NH_4HCO_3. The clear solution was loaded on a column of SEPHADEX™ G-100 ("SEPHADEX™ G-100" are beads for gel filtration preparation prepared by cross-linking dextran with epichlorohydrin, from Pharmacia, Baie d'Urfé, Qc) (1.5 x 90 cm) equilibrated in the same

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solvent. Fractions of 1.7 ml were collected and pooled into seven fractions (see Fig.3). A small portion of each was saved for estimating HCG equivalent activity and the remainder was lyophilized.

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Please amend pages 11 and 12 of the Disclosure as follows:

HCG receptor binding activity

A convenient test for HCG, a hormone which efficiently binds to the LH receptor, is to perform radioreceptor assays using membrane preparations of adult pig testes as described in detail (Sairam MR, 1983, *In*: *Hormonal Proteins and Peptides*. Li C. H., ed., pages 1-79; Manjunath P et al., 1982, *J Biol Chem* **257**: 7109-7115). Standard (CR-125 HCG from NICHD, Bethesda) or test samples were tested for ^{125}I-HCG binding as described (Sairam MR, 1983, *In*: *Hormonal Proteins and Peptides*. Li C. H., ed., pages 1-79). The total binding activity in each of the seven fractions was calculated and expressed as, ug HCG equivalent per fraction.

Steroidogenic activity

HCG is a highly potent steroidogenic hormone, therefore one reliable bioassay consists of incubating mouse Leydig tumour cells (MA-10, originally obtained from Dr. M. Ascoli, Iowa) with the test material as described (Sairam MR, 1983, *In*: *Hormonal Proteins and Peptides*. Li C. H., ed., pages 1-79). Progesterone in the medium was estimated by radioimmunoassay (Sairam MR, 1983, *In*: *Hormonal Proteins and Peptides*. Li C. H., ed., pages 1-79).

Electrophoretic Mobility Gel Shift Assay (EMSA)

Nuclear extracts were prepared from KSY-1 cell cultures according to the original procedure of Smeal (Smeal T et al., 1989,. *Genes Develop*. **3**:2091-2100). Binding reactions for AP-1 sites (TRE, TPA Response Element) were carried out as described (Smeal T et al., 1989,. *Genes Develop*. **3**:2091-2100, and reviewed in

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Saatcioglu F et al., 1994, *Semin. Cancer Biol.* 5:347-359). Synthetic collagenase TRE oligonucleotide probe of the sequence 5'-GGATCCGA**TGAGTCA**GCCA-3' (SEQ ID NO: 5) was end labelled with ^{32}P-ATP and EMSA performed as described (Smeal T et al., 1989,. *Genes Develop.* 3:2091-2100). Specificity was ascertained by using 100 molar excess of unlabelled TRE. The signal was quantified by phosphorimager analysis using the software by Molecular Dynamics (Sunnyvale, CA).

Pure HCG has no inhibitory activity in KS cells (Fig. 1)

Initial experiments were designed to confirm the inhibitory action of HCG. The effects on the two different KS cell lines were compared. In cells pre-treated with a commercial HCG preparation (Sigma or APL™) an inhibitory effect was elicited ($p < 0.05$) in all KS cell lines. In preliminary experiments a dose-dependent inhibition of cell growth was noted.

The two commercial HCG products (APL™ and Sigma) were tested, and near identical inhibition was obtained (Fig. 1), right-hand two bars). However, some HCG shipments were more potent than others.

Samples were used at an equivalent concentration of 50U/ml (Fig. 1). Note that upon treatment with Sigma-HCG (S) or Ayerst-HCG (APL™), KS cell growth was significantly reduced as compared with the vehicle-treated cells (C). In contrast, no inhibitory effect was noted using preparations of highly purified HCG. Legend: 1=dimeric HCG; 2=α HCG; 3=β HCG; 4=unrelated human urinary protein pool; *$p<0.05$.

Next, the anti-KS activity of a well characterized, pure dimeric HCG, pure α or β subunits and recombinant HCG was verified. Neither one of these

pure HCG's inhibited KS growth (Fig. 1, #1-3). The biological activity of these compounds was examined by induction of steroidogenesis in cultured Leydig cells. As expected, either recombinant or pure HCG elicited the classic biological responses, while neither α nor β HCG displayed any steroidogenic action.

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Molecular sieving of crude HCG (Fig. 2)

Generally, the pregnancy hormone ampouled into vials for clinical use is only about 25% pure for HCG as evaluated by biological activity and biochemical analyses (Manjunath P et al., 1982, *J Biol Chem* **257**: 7109-7115). The commercial HCG (APL™) was sorted into 7 distinct fractions using SEPHADEX™ chromatography (Fig. 2).

The contents of 3 vials of clinical grade APL™ (10,000 IU each) were dissolved in 0.05 M NH_4HCO_3 and subjected to molecular sieving on a column of SEPHADEX™ G-100 (1.5 x 90 cm). The eluted protein/peptide fractions monitored at A230 nm (panel E) were separated into seven pools identified as fraction pools # 1-7 on the X-axis. A total of 120 tubes (1.75 ml/tube) were collected. Lyophilized material in each pool was reconstituted in KS culture medium (without serum), and evaluated for cell proliferation (panel A). HCG receptor binding in pig testicular membranes (panel B) and steroidogenic activity in MA-10 cells (panel C) were determined. Panel D: bar graphs show quantitative densitometric scanning of AP-1 binding and insert shows the actual EMSA protein-DNA complexes of fractions (fr) 2,4 and 7; nd=not determined. KSY-1 cells were treated with the indicated reagents at an equivalent concentration 100 U/ml for 4 days. Note clear segregation of HCG hormone activity on gonadal cells (pool 2) and inhibitory action on KS cells (pool 7) *$p < 0.05$.

Over 85 % HCG receptor binding activity (Fig. 2B) was recovered in the first two pooled fractions where high molecular weight proteins of the

size of pure HCG would emerge. The Ve/Vo ratio of the early major fraction (pool #2) corresponded to bona fide HCG. These fractions may also contain the hormone

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subunits (α/β) or their degraded products in addition to other unidentified materials present in the crude extract. Fraction #7 consists, as shown in previous studies (Sairam MR, 1983, *In: Hormonal Proteins and Peptides*. Li C. H., ed., pages 1-79), of relatively small peptides along with other agents present in the APL™ formulation. Either the steroidogenic or the binding activity that is characteristic of HCG (but not its subunits) was highest in the 2nd fraction (Figs. 2B and C). These results are consistent with receptor binding assays in which only the dimeric (α-β combined) HCG but not the individual subunits or their cleaved products are biologically active (Sairam MR, 1983, *In: Hormonal Proteins and Peptides*. Li C. H., ed., pages 1-79; Manjunath P et al., 1982, *J Biol Chem* **257**: 7109-7115). On the contrary, only fraction #7 contained KS inhibitory activity.

Down-regulation of AP-1 binding by HCG components (Fig. 3)

Activating protein-1 (AP-1) is a transcriptional activator which is induced by 12-O-tetradecanyl phorbol-13-acetate (TPA) tumor promoter, several growth factors and various extracellular stimuli (reviewed in Saatcioglu F et al., 1994, *Semin. Cancer Biol.* **5**:347-359). AP-1 consists of proteins of jun and fos families which associate to form homo-(jun/jun) or heterodimers (jun/fos) and recognize a consensus sequence 5'-TGA G/C TCA-3' known as TPA Response Element (TRE) present on AP-1 regulated genes. AP-1 complexes are considered to play important roles in several signal transduction pathways such as growth

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stimulation, differentiation, neuronal excitation and transformation (Saatcioglu F et al., 1994, *Semin. Cancer Biol.* **5**:347-359). APL™-HCG and components in fraction 7 significantly inhibited AP-1 binding to TRE

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Please amend pages 15 and 16 of the Disclosure as follows:

in KSY-1 cells (Fig. 2D). APL™-HCG inhibited AP-1 binding by 1.5, 3 and 2 fold respectively after 3, 6 and 12 hours of treatment (Fig. 3).

Cells were incubated with 50 IU/ml APL™-HCG (+) or with vehicle (-) for the indicated time periods (Fig. 3). Nuclear extracts were prepared and EMSA was performed. Results shown are representative of three experiments. Arrow-head points to free probe. 100ex.= 100 fold excess unlabelled probe. Top shows the actual gel shifts while bottom panel provides quantitative phosphorimager measurement of the major band (arrow); * denotes $p < 0.05$ as compared to vehicle-treated.

A dose-response was also observed with near maximal effect noted at approximately 100 IU/ml. Therefore, repression of AP-1 may be an important pathway by which inhibition of KSY-1 cells occurs.

Purification of the HIP using reversed phase-HPLC (Fig. 4)

APL™ was purchased from Wyeth-Ayerst Cat. # DIN 02168936 and shipped in an insulated box packed with refrigerant. Upon receipt, APL™ was stored at 4°C. One APL™ vial (which contained the dried product) lot # JA(L)3YYF-AB was reconstituted with one (1) ml of the solvent sold with the APL™ ampoule at room temperature and processed for HPLC within one hour. The powder was readily dissolved resulting in a homogeneous "solution". This "solution" was injected into a Waters™ HPLC apparatus fitted with a 7.8 x 300 mm C-18™ column ("C-18™ column" is a Reverse-phase chromatographic column with bonded phase containing n-octyldecyl chains, available from Waters, Corporation). Elution from the column was done using an increasing linear

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isocratic gradient of acetonitrile in water containing 0.1 % trifluoroacetic acid. The gradient was increased from 5% to 75% acetonitrile. The absorbaency was monitored at 220 wavelength during the elution and fractions were collected manually in siliconized polypropylene tubes. When regular (i.e. non-siliconized) tubes were used it was later found that biological activity was lost. After collection, the fractions were immediately placed in a Savant™ Speed-vac apparatus in order to dry the samples. The gradient is drawn on Fig. 4; the right-side or Y axis shows the % acetetonile (%B; B: 80% acetonitrile in water containing 0.1% trifluoroacetic acid) and the X axis indicates time, in minutes. The absorbency at 220 nm was recorded and recorded on the Y axis. The two peaks (D & E) indicated by arrows were subsequently found (see Fig. 5 below) to contain the KS inhibitory activity.

Bioassay of the collected fractions following HPLC separation (Fig. 5)

The fractions (peaks) indicated by arrows on Fig. 4. were lyophilized and each was reconstituted in one (1) ml of RPMI culture medium (without serum) and tested for biological activity using the KS-Y1 cells. Since the original material was supplied as 10 000 IU of HCG, by analogy, it was assumed arbitrarily that one of the fractions should contain arbitrarily 10,000 IU of anti-KS activity. With such an assumption, the doses were evaluated throughout the present application. The biological activity was tested in absence (0) or presence of different doses (10, 100 & 200 IU/ml). The fraction indicated as "mix" represents one pool made by mixing equivalent amounts of fractions A-E. It can be

seen from Fig. 5 that fractions D, E and "Mix" display an inhibitory activity.

Analysis of the active fractions (HIP) by MALDI-TOF mass spectrometry (Figs. 6 and 7)

Briefly, an aliquot of each sample was embedded in a low molecular weight UV-absorbing matrix (α-cyano-4-hydroxycinnamic acid) to enhance sample ionization and then subjected to MALDI-TOF (Matrix Assisted Laser